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Short communication

Synthesis and biological evaluation of new 4*H*-pyrano[2,3-*b*]quinoline derivatives that block acetylcholinesterase and cell calcium signals, and cause neuroprotection against calcium overload and free radicals

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Abstract

The synthesis and biological evaluation of ethyl 5-amino-4-(3-pyridyl)-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrano[2,3-*b*]quinoline-3-carboxylates (**9**–**11**) is described. We have found that these compounds inhibit AChE with a mild potency, mitigates the $[Ca^{2+}]_c$ triggered by high K^+, and cause neuroprotection against Ca^{2+} overloading and free radical-induced neuronal death.

Keywords: Tacrine; Pyrano[2,3-*b*]quinolines; Inhibitors; AChE; BuChE; Neuroprotection; Voltage-dependent calcium channels (VDCC); Alzheimer's disease; Multipotent drugs

1. Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders, affecting around 7% of the population above 65 years [1]. During the past two decades, AChE inhibitors (AChEI) tacrine (**1**) (Fig. 1), donepezil, and rivastigmine [2] have become the only available drugs for the treatment of AD. However, the multifactorial pathogenesis of AD suggests that molecules with two or more mechanisms of action, acting in a complementary manner, could be more efficacious for patients suffering the disease.

A few years ago we started a project aimed at the synthesis and the pharmacological study of new tacrine analogues with additional properties. We were particularly interested in multipotent drugs behaving as AChEI and modulators of voltage-dependent Ca^{2+} channels (VDCCs) [3–5].

In this context, we have recently synthesized and evaluated a number of ethyl 5-amino-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrano[2,3-*b*]quinoline-3-carboxylates of type **A** (Fig. 1) that were acceptable AChE inhibitors, a few of them possessing a remarkable cytoprotective effect against cell death caused by Ca^{2+} overload in a model of bovine chromaffin cells [6]. For further developments, and based on the better neuroprotective results obtained against veratridine and the AChE inhibition activity with compound **2** (Fig. 1) [6], next we considered the synthesis of compounds containing a 3-pyridyl ring moiety at C-4, with substituents (X = methoxy, bromo and chloro) at C-2′, and a fused cyclohexane ring. In this work we report the synthesis and biological evaluation of the new ethyl 5-amino-2-methyl-4-(3-pyridyl)-6,7,8,9-tetrahydro-4*H*-pyrano [2,3-*b*]quinoline-3-carboxylates (**9**–**11**) (Scheme 1).

2. Results and discussion

Starting from commercially available 3-pyridinecarboxaldehydes (**3**–**5**), 4*H*-pyrans **6**–**8** were obtained uneventually

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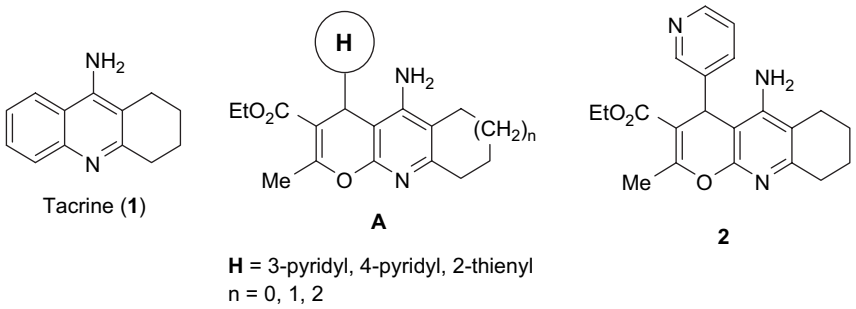


Fig. 1. Structure of tacrine (1) and some previously ethyl 4H-pyranol [2,3-b] quinoline 3-carboxylates **(A**,**2)** synthesized in our laboratory [3,6].

[7]. As expected, after treatment of these intermediates with cyclohexanone, in the presence of aluminium trichloride, a successful Friedländer reaction [8] resulted, affording the target molecules **9**–**11** in good yields (Scheme 1).

To determine the potential interest of compounds **9**–**11** for the treatment of AD, their AChE and butyrylcholinesterase (BuChE) inhibitory activity was assayed according to standard methodology [9,10]. Comparing with tacrine (**1**), the newly synthesized compounds are less potent (Table 1). Compound **10**, with a 2-bromo substituent in the 3-pyridyl ring, was 10-fold less active than tacrine, but the most potent in the group. Comparing with the reference compound **2** [6], tacrines **9**–**11** showed a potentiated activity, compound **10**, for instance, being 4-fold more potent. Regarding the BuChE activity, compounds **9**–**11** inhibited more strongly AChE than BuChE, the most active derivative being compound **9**, which was the less active inhibitor of AChE of the new derivatives synthesized.

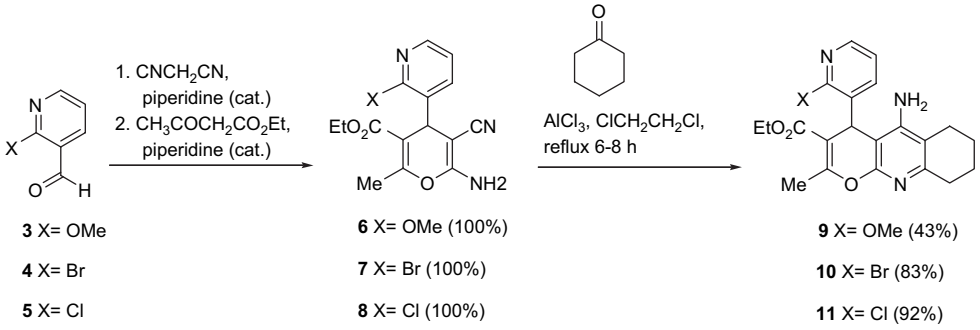
In this context, we considered of interest to find out if some of our compounds were binding the peripheral anionic site (PAS). Thus, a propidium competition assay [11] was carried out at the concentrations of 0.3 and 3 μM. Very interestingly, compounds **9** and **11** inhibited fluorescence about 30% and 20%, respectively, at the concentration of 3 μM. Note also that the new compounds were more effective than tacrine or donepezil in displacing propidium from the PAS of AChE (Table 2), and that the most potent AChEI, compound **10**, was unable to displace propidium in the PAS, suggesting that most probably this inhibitor is binding the catalytic center of AChE.

Regarding the neuroprotection, two different toxic stimuli, veratridine and a solution with high concentration of KCl (70 mM) that cause cell death by Ca^{2+} overload, were used in our experiments.

As shown in Tables 3 and 4, compounds **9**–**11** showed a marked neuroprotective profile, all of them being much more potent than tacrine. Compound **10**, the best AChEI, has also proven to be the best neuroprotector, with values around 40–46%, in both experiments.

Oxidative damage appears to play also an important role in the progressive neuronal death characteristic of AD [12,13]. For that reason, we considered the convenience of studying the possible antioxidant effect of our compounds on SH-SY5Y neuroblastoma cells exposed to 60 μM H_2O_2. Results are shown in Table 5. Although all compounds showed a remarkable neuroprotective effect, compound **11**, was especially potent against toxicity induced by H_2O_2.

Finally, and since a significant cytoprotection was achieved with all compounds against Ca^{2+} overload induced by 70 mM K^+ (see Table 4), we decided to study a possible blockade of VDCC by these compounds, which could be promoted by preventing excess Ca^{2+} entry to the cells. All compounds were tested at the concentration of 3 μM for chromaffin cells and 0.3 μM for SH-SY5Y cells (Table 6). The % blockade exerted by the compounds in chromaffin cells, which posses around 20% of VDCC of the L-subtype, suggests that they are probably acting on such type of calcium channels. This is consistent with the fact that in neuroblastoma cells, where the majority of Ca^{2+} channels belong to the L-subtype, the blockade is much higher



Scheme 1. Synthesis of the new ethyl 4H-pyranol [2,3-b] quinoline 3-carboxylates **[9**–**11]**.

Table 1
AChE and BuChE IC_{50} (μM) data for the inhibitory activity of compounds (**1**, **2**, **9**–**11**)

	AChE IC_{50} (μM)	BuChE IC_{50} (μM)	Ratio of BuChE/AChE
Tacrine (**1**)	0.14	0.04	0.29
2 [6]	7.11	13	1.8
9	3.92	62.4	15.91
10	1.86	>100	>53.76
11	2.14	>100	>46.72

Data are expressed as the means ± S.E.M. of at least three different experiments in quadruplicate. nc = not calculated.

Table 3
Cell viability of bovine chromaffin cells, expressed as % LDH released in the presence of veratridine

Compound (3 μM)	LDH (% vehicle)	% Protection
Vehicle	100	
Tacrine (**1**)	98.7 ± 2.9	1.3
9	64.75 ± 2.03***	40.9
10	60.08 ± 1.3***	46.7
11	61.46 ± 1.3***	45.24

Data are expressed as the means ± S.E.M. of at least three different cultures in quadruplicate. *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$ with respect to a control without any drug. ns = not significant.

in general. As shown, although less potent than nimodipine, compounds **9**–**11** blocked VDCC much efficiently than tacrine.

3. Conclusions

To sum up, in this work we have described the synthesis and extensive pharmacological evaluation of a series of known and new ethyl 5-amino-4-(3-pyridyl)-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrano[2,3-*b*]quinoline-3-carboxylates (**9**–**11**).

The newly synthesized compounds are in general better inhibitors of AChE and more selective for this enzyme than compounds of type (**A**) (Fig. 1) [6]. It is also worth of mention that comparing with the reference compound **2** [6], tacrines **9**–**11** showed a potentiated activity, due to the substitution at C-2′ in the 3-pyridyl ring system. Note also that the most potent AChEI, compound **10**, was unable to displace propidium in the PAS, suggesting that most probably this inhibitor is binding the catalytic center of the AChE. We had studied neuroprotection in a cellular model of Ca^{2+} overload caused by veratridine. Our experiments indicate that the compounds could be acting on the L-subtype of VDCC, as the percentage of blockade in chromaffin cells (around 20%) and in neuroblastoma cells (about 35–40%) suggests. As far as neuroprotection is concerned, in the model of Ca^{2+} overload induced by veratridine, compounds **9**–**11** show a good neuroprotective profile. In the potassium-induced Ca^{2+} overload, the protection is good for all the compounds. Compound **11** was the less neuroprotectant, with only 23% of cell-death suppression.

Table 2
Displacement of propidium iodide from the peripheral anionic site by compounds tacrine (**1**), donepezil, and **9**–**11**, measured as the inhibition of the fluorescence of propidium bound to AChE

Compound	% Inhibition (0.3 μM)	% Inhibition (3 μM)
Tacrine (**1**)	3.94 ± 1.8	5.97 ± 1.4
Donepezil	18.6 ± 6.3	12.2 ± 11.7
9	18.7 ± 3.4 ns	28.3 ± 3.3***
10	13.4 ± 4.1 ns	19.3 ± 4.0 ns
11	21.2 ± 4.8*	20.2 ± 4.5*

Data are expressed as the means ± S.E.M. of at least three different experiments in quadruplicate. *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$ with respect to propidium bound to AChE. ns = not significant.

Unexpectedly compound **10**, that only differs from **11** in the type of substituent (a bromine atom instead of a chlorine) attached at the 3-pyridyl moiety located at C-4, afforded the best neuroprotection properties (2-fold better than **11**). To sum up, these data are very interesting, and suggest future structural modifications for activity improvement.

4. Experimental part

4.1. General methods

Reactions were monitored by TLC using precoated silica gel aluminium plates containing a fluorescent indicator (Merck, 5539). Detection was done by UV (254 nm) followed by charring with sulfuric–acetic acid spray, 1% aqueous potassium permanganate solution or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during work-ups and the removal of solvents was carried out under *vacuum* with a rotary evaporator. Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were obtained on a Perkin–Elmer Spectrum One spectrophotometer. ^{1}H NMR spectra were recorded with a Varian VXR-200S spectrometer, using tetramethylsilane as internal standard and ^{13}C NMR spectra were recorded with a Bruker WP-200-SY. All the assignments for protons and carbons were in agreement with 2D COSY, HSQC, HMBC, and 1D NOESY spectra. Values with (*) can be interchanged. Elemental analyses were conducted on a Carlo Erba EA 1108 apparatus.

Table 4
Cell viability in the human neuroblastoma cell line SH-SY5Y, expressed as increase of LDH released in the presence of 70 mM K^+

Compound (0.3 μM)	LDH release (% vehicle)	% Protection
Vehicle	100	–
Tacrine (**1**)	90.4 ± 4.1 ns	13.4
9	69.19 ± 0.82***	38.87
10	66.93 ± 0.92***	41.75
11	1.63 ± 1.62**	23.12

Data are expressed as the means ± S.E.M. of at least three different cultures in quadruplicate. *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$ with respect to a control without any drug. ns = not significant.

Table 5
Cell viability expressed as increase of LDH released by human neuroblastoma cells in the presence of 60 μM H_2O_2

Compound (0.3 μM)	LDH release (% vehicle)	% Protection
Vehicle	100	–
Tacrine (**1**)	98.0 ± 1.80 ns	0.8
9	58.1 ± 1.4***	48.73
10	64.6 ± 1.4***	41.19
11	48.6 ± 2.2***	61.18

Data are expressed as the means ± S.E.M. of at least three different cultures in quadruplicate; ns = not significant. *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$ with respect to a control without any drug.

4.2. General method for the synthesis of 6-amino-5-cyano-4-aryl-2-methyl-4H-pyrans

To a solution of the corresponding aldehyde (1 equiv) in methanol (0.24 M), under argon, malononitrile (1.1 equiv) and a catalytic amount of piperidine were added. The mixture was stirred at rt for 15–20 min. Then, ethyl acetoacetate (1.1 equiv) and some drops of piperidine (cat.) were added. The mixture was stirred at rt for 15–20 min, and the precipitated solid was isolated by filtration, washed with cold methanol, dried and recrystallized.

4.2.1. Ethyl 6-amino-5-cyano-4-(2-methoxy-3-pyridyl)-2-methyl-4H-pyran-3-carboxylate (**6**)

Following the General method, 2-methoxy-3-pyridinecarboxaldehyde (**3**) (500 mg, 3.64 mmol), malononitrile (265 mg, 4.01 mmol), ethyl acetoacetate (521.3 mg, 4.01 mmol), piperidine (10 drops), and methanol (15 mL), after 15 min, gave compound (**6**) (1.1 g, 100%): mp 175–177 °C; IR (KBr) ν 3401, 3326, 3217, 2978, 2949, 2188, 1690, 1605, 1466, 1415, 1370, 1333, 1261, 1214, 1180 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.01 (dd, $J_{4'\text{-}5'} = 6.9$ Hz, $J_{4'\text{-}6'} = 1.7$ Hz, 1 H, H4′), 7.40 (dd, $J_{6'\text{-}4'} = 1.7$ Hz, $J_{6'\text{-}5'} = 5.5$ Hz, 1 H, H6′), 6.92 (dd, $J_{5'\text{-}4'} = 6.9$ Hz, $J_{5'\text{-}6'} = 5.5$ Hz, 1 H, H6′), 6.84 (s, 2 H, NH_2), 4.56 (s, 1 H, H4), 3.92 (q, $J = 7.12$ Hz, 2 H, $CO_2CH_2CH_3$), 3.84 (s, 3 H, CH_3O), 2.30 [s, 3 H, $CH_3C(2)$], 0.96 (t, $J = 7.12$ Hz, 3 H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 165.3 (C═O), 160.7 (C2′), 159.1 (C6), 157.9 (C2), 145.0 (C4′), 137.2 (C6′), 126.8 (C1′), 119.5 (C≡N), 117.1 (C5′), 105.3 (C3), 60.0 (OCH_2CH_3), 55.3 (C5), 53.1 (CH_3O), 32.8 (C4), 18.1 [CH_3(C2)], 13.6 (CH_3CH_2O); MS (APCI+) *m/z* $[M + 1]^+$ 316.3 $[M + Na]^+$ 338.3; $[2M + Na]^+$ 653.5. Anal. Calcd. for $C_{16}H_{17}N_3O_4$: C, 60.94; H, 5.43; N, 13.33. Found: C, 61.21; H, 5.70; N, 13.47.

Table 6
Effect of the different compounds on the increase of $[Ca^{2+}]_c$ elicited by 70 mM K^+ in chromaffin and SH-SY5Y cells (% inhibition with respect to a control without any drug)

Compound	% Inhibition of chromaffin cells (3 μM)	% Inhibition of neuroblastoma cells (0.3 μM)
Tacrine (**1**)	0	0
Nimodipine	25.0 ± 2.7***	78.89 ± 2.7***
9	23.0 ± 2.2***	33.4 ± 2.5***
10	24.5 ± 2.4***	43.4 ± 3.9***
11	20.3 ± 2.4***	33.0 ± 3.1***

Data are expressed as the means ± S.E.M. of at least three different cultures in quadruplicate. *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$ with respect to a control without any drug.

4.2.2. Ethyl 6-amino-4-(2-bromo-3-pyridyl)-5-cyano-2-methyl-4H-pyran-3-carboxylate (**7**)

Following the General method, 2-bromo-3-pyridinecarboxaldehyde (**4**) (500 mg, 2.69 mmol), malononitrile (188.76 mg, 2.86 mmol), ethyl acetoacetate (371.8 mg, 2.86 mmol), piperidine (15 drops), and methanol (15 mL), after 20 min, gave compound (**7**) (970 mg, 100%): mp 200–201 °C; IR (KBr) ν 3394, 3311, 3151, 2194, 1704, 1666, 1609, 1402, 1378, 1321, 1268, 1222, 1116 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.23 (dd, $J_{4'\text{-}5'} = 4.5$ Hz, $J_{4'\text{-}6'} = 1.9$ Hz, 1 H, H4′), 7.63 (dd, $J_{6'\text{-}4'} = 1.9$ Hz, $J_{6'\text{-}5'} = 7.6$ Hz, 1 H, H6′), 7.41 (dd, $J_{5'\text{-}4'} = 4.5$ Hz, $J_{5'\text{-}6'} = 7.6$ Hz, 1 H, H6′), 7.03 (s, 2 H, NH_2), 4.80 (s, 1 H, H4), 3.92 (q, $J = 7.1$ Hz, 2 H, $CO_2CH_2CH_3$), 2.34 [s, 3 H, CH_3(C2)], 0.95 (t, $J = 7.1$ Hz, 3 H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 165.2 (C═O), 158.9 (C2*), 158.8 (C6*), 149.0 (C4′), 142.4 (C1′), 138.9 (C6′), 124.2 (C5′), 119.2 (C≡N), 105.6 (C3), 60.5 (OCH_2CH_3), 55.6 (C5), 37.7 (C4), 18.5 [CH_3(C2)], 14.0 (CH_3CH_2O); MS (APCI+) *m/z* $[M + 1]^+$ 387.0 $[M + Na]^+$ 410.0; $[2M + Na]^+$ 753.3. Anal. Calcd. for $C_{15}H_{14}BrN_3O_3$: C, 49.47; H, 3.87; N, 11.54. Found: C, 49.35; H, 3.71; N, 11.63.

4.2.3. Ethyl 6-amino-4-(2-chloro-3-pyridyl)-5-cyano-2-methyl-4H-pyran-3-carboxylate (**8**)

Following the General method, 2-chloro-3-pyridinecarboxaldehyde (**5**) (500 mg, 3.53 mmol), malonodinitrile (256.1 mg, 3.88 mmol), ethyl acetoacetate (504 mg, 3.88 mmol), piperidine (15 drops), and methanol (15 mL), after 15 min, gave compound (**8**) (1.1 g, 100%): mp 180–183 °C; IR (KBr) ν 3373, 3304, 3152, 2992, 2971, 2189, 1724, 1675, 1607, 1410, 1226 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.27 (dd, $J_{4'\text{-}5'} = 4.5$ Hz, $J_{4'\text{-}6'} = 1.7$ Hz, 1 H, H4′), 7.68 (dd, $J_{6'\text{-}4'} = 1.7$ Hz, $J_{6'\text{-}5'} = 7.6$ Hz, 1 H, H6′), 7.39 (dd, $J_{5'\text{-}4'} = 4.5$ Hz, $J_{5'\text{-}6'} = 7.6$ Hz, 1 H, H6′), 7.03 (s, 2 H, NH_2), 4.80 (s, 1 H, H4), 3.91 (q, $J = 7.2$ Hz, 2 H, $CO_2CH_2CH_3$), 2.33 [s, 3 H, CH_3(C2)], 0.94 (t, $J = 7.2$ Hz, 3 H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 164.9 (C═O), 158.6 (C2*), 158.5 (C6*), 148.8 (C2′), 148.5 (C4′), 139.0 (C6′), 138.7 (C1′), 123.8 (C5′), 118.9 (C≡N), 104.9 (C3), 60.1 (OCH_2CH_3), 55.0 (C5), 35.6 (C4), 18.2 [CH_3(C2)], 13.5 (CH_3CH_2O); MS (APCI+) *m/z* $[M + 1]^+$ 320.0 $[M + Na]^+$ 342.3. Anal. Calcd. for $C_{15}H_{14}N_3O_3Cl$: C, 56.35; H, 4.41; N, 13.14. Found: C, 56.06; H, 4.33; N, 13.14.

4.3. General method for the Friedländer reaction

Aluminium chloride (1.2–1.7 equiv) was suspended in dry 1,2-dichloroethane (0.15 M) at rt, under argon. The corresponding 4*H*-pyran (1 equiv) and cyclohexanone (1.2–1.7 equiv)

were added. The reaction mixture was refluxed for 6.5–8.5 h. When the reaction was over (TLC analysis), a mixture of THF/H_2O (1:1) was added at rt, and aqueous solution of sodium hydroxide (10%) was added dropwise to the mixture until the aqueous solution was basic. After stirring for 30 min, the mixture was extracted three times with dichloromethane. The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered and the solvent was evaporated. The resultant solid was purified by silica gel flash chromatography using methanol/dichloromethane mixtures of increasing polarities as eluent to give pure compounds.

*4.3.1. Ethyl 5-amino-4-(2-methoxy-3-pyridyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrano [2,3-b]quinoline-3-carboxylate (**9**)*

Following the General method, from compound **6** (250 mg, 0.79 mmol), $AlCl_3$ (157.6 mg, 1.18 mmol), $ClCH_2CH_2Cl$ (5 mL), and cyclohexanone (116.2 mg, 1.18 mmol) after 6.5 h, product **9** (136 mg, 43%) was obtained: mp 158–160 °C; IR (KBr) ν 3455, 3391, 3355, 2930, 1712, 1638, 1570, 1447, 1401, 1378, 1217 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.99 (dd, $J_{4'\text{-}6'} = 1.9$ Hz, $J_{4'\text{-}5'} = 4.9$ Hz, 1 H, H4′), 7.53 (dd, $J_{6'\text{-}4'} = 1.9$ Hz, $J_{6'\text{-}5'} = 7.3$ Hz, 1 H, H6′), 6.92 (dd, $J_{5'\text{-}4'} = 4.9$ Hz, $J_{5'\text{-}6'} = 7.3$ Hz, 1 H, H5′), 5.52 (s, 2 H, NH_2), 5.04 (s, 1 H, H4), 3.94 (q, $J = 7.1$ Hz, 2 H, OCH_2CH_3), 3.92 (s, 3 H, CH_3O), 2.39 [s, 3 H, CH_3(C2)], 2.20–1.85 (m, 4 H, 2 H6, 2 H9), 1.65 (br s, 4 H, 2 H7, 2 H8), 1.04 (t, $J = 7.4$ Hz, 3 H, OCH_2CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.3 (C=O), 161.2 (C2′), 160.0 (C2), 154.1 (C9a), 152.7 (C10a), 151.3 (C5), 145.4 (C4′), 139.1 (C6′), 127.3 (C1′), 118.3 (C5′), 113.2 (C5a), 105.1 (C3), 98.0 (C4a), 60.2 (OCH_2CH_3), 53.8 (CH_3O), 32.3 (C4), 31.2 (C6), 23.3 (C9), 22.7 (C7), 22.5 (C8), 19.4 [CH_3(C2)], 14.2 (CH_3CH_2O); MS (APCI+) m/z $[M + 1]^+$ 396.3; $[2M + Na]^+$ 813.5. Anal. Calcd. for $C_{22}H_{25}N_3O_4$: C, 66.82; H, 6.37; N, 10.63. Found: C, 66.37; H, 6.05; N, 10.72.

*4.3.2. Ethyl 5-amino-4-(2-bromo-3-pyridyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrano[2,3-b]quinoline-3-carboxylate (**10**)*

Following the General method, from compound **7** (250 mg, 0.69 mmol), $AlCl_3$ (137.65 mg, 1.03 mmol), $ClCH_2CH_2Cl$ (5 mL), and cyclohexanone (101.4 mg, 1.03 mmol) after 8 h, product **10** (255 mg, 83%) was obtained: mp 153–155 °C; IR (KBr) ν 3455, 3391, 3355, 2930, 1712, 1638, 1570, 1447, 1401, 1378, 1217 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.21 (dd, $J_{4'\text{-}6'} = 1.9$ Hz, $J_{4'\text{-}5'} = 4.6$ Hz, 1 H, H4′), 7.78 (dd, $J_{6'\text{-}4'} = 1.9$ Hz, $J_{6'\text{-}5'} = 7.7$ Hz, 1 H, H6′), 7.40 (dd, $J_{5'\text{-}4'} = 4.6$ Hz, $J_{5'\text{-}6'} = 7.7$ Hz, 1 H, H5′), 5.45 (s, 2 H, NH_2), 5.12 (s, 1 H, H4), 4.06 (q, $J = 7.4$ Hz, 2 H, $CO_2CH_2CH_3$), 2.54 (m, 2 H, 2 H9), 2.40 [s, 3 H, CH_3(C2)], 2.20 (m, 2 H, H6), 1.69 (br s, 4 H, 2 H7, 2 H8), 1.13 (t, $J = 7.4$ Hz, 3 H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.0 (C=O), 160.9 (C9a), 153.9 (C10a), 153.3 (C2), 151.4 (C5), 149.0 (C4′), 141.7 (C1′), 141.0 (C6′), 140.4 (C2′), 124.4 (C5′), 113.6 (C5a), 104.8 (C3), 97.2 (C4a), 60.3 (OCH_2CH_3), 37.2 (C4), 32.2 (C6), 23.2 (C9), 22.5 (C7), 22.2 (C8), 19.5 [CH_3, CH_3(C2)], 14.4 (CH_3CH_2O); MS (APCI+) m/z $[M + 1]^+$ 445.0, 446.0; $[2M + Na]^+$ 911.2, 914.1. Anal. Calcd. for $C_{21}H_{22}BrN_3O_3$: C, 56.77; H, 4.99; N, 9.46. Found: C, 56.41; H, 4.91; N, 9.30.

*4.3.3. Ethyl 5-amino-4-(2-chloro-3-pyridyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrano [2,3-b]quinoline-3-carboxylate (**11**)*

Following the General method, from compound **8** (250 mg, 0.78 mmol), $AlCl_3$ (155.61 mg, 1.17 mmol), $ClCH_2CH_2Cl$ (5 mL), and cyclohexanone (134.15 mg, 1.17 mmol) after 7.5 h, product **11** (288 mg, 92%) was obtained: mp 196–198 °C; IR (KBr) ν 3437, 3318, 3197, 2963, 2929, 2855, 1724, 1647, 1600, 1573, 1447, 1406, 1376, 1220 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.21 (dd, $J_{4'\text{-}6'} = 1.9$ Hz, $J_{4'\text{-}5'} = 4.6$ Hz, 1 H, H4′), 7.78 (dd, $J_{6'\text{-}4'} = 1.9$ Hz, $J_{6'\text{-}5'} = 7.7$ Hz, 1 H, H6′), 7.40 (dd, $J_{5'\text{-}4'} = 4.6$ Hz, $J_{5'\text{-}6'} = 7.7$ Hz, 1 H, H5′), 5.45 (s, 2 H, NH_2), 5.12 (s, 1 H, H4), 4.06 (q, $J = 7.4$ Hz, 2 H, $CO_2CH_2CH_3$), 2.54–2.20 (m, 4 H, 2 H6, 2 H9), 2.40 [s, 3 H, CH_3(C2)], 1.69 (br s, 4 H, 2 H7, 2 H8), 1.13 (t, $J = 7.4$ Hz, 3H, $CO_2CH_2CH_3$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 166.0 (C=O), 160.9 (C9a), 153.9 (C10a), 153.3 (C2), 151.4 (C5), 149.0 (C4′), 141.7 (C1′), 141.0 (C6′), 140.4 (C2′), 124.4 (C5′), 113.6 (C5a), 104.8 (C3), 97.2 (C4a), 60.3 (OCH_2CH_3), 37.2 (C4), 32.2 (C6), 23.2 (C9), 22.5 (C7), 22.2 (C8), 19.5 [CH_3, CH_3(C2)], 14.4 (CH_3CH_2O); MS (APCI+) m/z $[M + 1]^+$ 400.1 $[M + Na]^+$ 422.1; $[2M + Na]^+$ 821.3. Anal. Calcd. for $C_{21}H_{22}ClN_3O_3$: C, 63.08; H, 5.55; N, 10.51. Found: C, 62.97; H, 5.45; N, 10.47.

4.4. Preparation and culture of bovine chromaffin cells

Bovine adrenal medullary chromaffin cells were isolated using collagenase digestion and Percoll gradients, as previously described [14]. Cells were suspended in Dulbecco's Modified Eagle's Medium, DMEM (GIBCO, Madrid, Spain), supplemented with 5% foetal calf serum, 50 U/mL penicillin and 50 μg/mL streptomycin (GIBCO, Madrid, Spain). The cells were pre-plated for 30 min and proliferation inhibitors (10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 10 μM leucine methyl ester) were added to the medium to prevent excessive growth of fibroblasts that would interfere with chromaffin cell-death measurements; 5×10^5 cells/well were plated in 24-well dishes. Cultures were maintained in an incubator for 2–4 days at 37 °C in a water-saturated atmosphere with 5% CO_2. Cell treatments were performed in DMEM, free of serum and inhibitors of cell proliferation.

4.5. Culture of SH-SY5Y cells

SH-SY5Y cells, at passages between 3 and 16 after de-freezing, were maintained in a DMEM containing 15 nonessential amino-acids (NEAAs) and supplemented with 10% foetal calf serum (FCS), 1 mM glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin (reagents from GIBCO, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in 5% CO_2/humidified

air. Stock cultures were passaged 1:4 twice weekly. For assays, SH-SY5Y cells were subcultured in 24-well plates at a seeding density of 2×10^5 cells/well, or in 96-well plates at a seeding density of 8×10^4 cells/well. For the cytotoxicity experiments cells were treated with drugs before confluence, in DMEM free of serum.

4.6. Measurement of lactic dehydrogenase (LDH) activity

Extracellular and intracellular LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death kit (Roche–Boehringer, Mannheim, Germany) according to the manufacturer's indications. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; released LDH was defined as the percentage of extracellular compared to total LDH activity. Data were expressed as the means ± S.E.M. of at least three different cultures in quadruplicate. LDH released was calculated for each individual experiment considering 100% the extracellular LDH released by the vehicle with respect to the total. To calculate % protection, LDH release was normalized as follows: in each individual triplicate experiment, LDH release obtained in non-treated cells (basal) was subtracted from the LDH released upon H_2O_2 treatment and normalized to 100% and that value was subtracted from 100.

4.7. Measurement of cytosolic Ca^{2+} concentrations [Ca^{2+}]

For these experiments, SH-SY5Y neuroblastoma cells were grown at confluence in 96-well black dishes. Cells were loaded with 4 μM fluo 4/AM for 1 h at 37 °C in DMEM. Then cells were washed twice with Krebs–Hepes solution and kept at room temperature for 30 min before the beginning of the experiment. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Optima, BMG, Germany). Wavelengths of excitation and emission were 485 and 520 nm, respectively.

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